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Published in:
The Journal of Biological Chemistry

DOI:
[10.1074/jbc.M311966200](https://doi.org/10.1074/jbc.M311966200)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Jong, R. M., Brugman, W., Poelarends, G. J., Whitman, C. P., & Dijkstra, B. W. (2004). The X-ray structure of trans-3-chloroacrylic acid dehalogenase reveals a novel hydration mechanism in the tautomerase superfamily. *The Journal of Biological Chemistry*, 279(12), 11546 - 11552.
<https://doi.org/10.1074/jbc.M311966200>

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The X-ray Structure of *trans*-3-Chloroacrylic Acid Dehalogenase Reveals a Novel Hydration Mechanism in the Tautomerase Superfamily*

Received for publication, October 31, 2003, and in revised form, December 23, 2003
Published, JBC Papers in Press, December 28, 2003, DOI 10.1074/jbc.M311966200

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Isomer-specific 3-chloroacrylic acid dehalogenases function in the bacterial degradation of 1,3-dichloropropene, a compound used in agriculture to kill plant-parasitic nematodes. The crystal structure of the heterohexameric *trans*-3-chloroacrylic acid dehalogenase (CaaD) from *Pseudomonas pavonaceae* 170 inactivated by 3-bromopropiolate shows that Glu-52 in the α -subunit is positioned to function as the water-activating base for the addition of a hydroxyl group to C-3 of 3-chloroacrylate and 3-bromopropiolate, whereas the nearby Pro-1 in the β -subunit is positioned to provide a proton to C-2. Two arginine residues, α Arg-8 and α Arg-11, interact with the C-1 carboxylate groups, thereby polarizing the α,β -unsaturated acids. The reaction with 3-chloroacrylate results in the production of an unstable halohydrin, 3-chloro-3-hydroxypropanoate, which decomposes into the products malonate semialdehyde and HCl. In the inactivation mechanism, however, malonyl bromide is produced, which irreversibly alkylates the β Pro-1. CaaD is related to 4-oxalocrotonate tautomerase, with which it shares an N-terminal proline. However, in 4-oxalocrotonate tautomerase, Pro-1 functions as a base participating in proton transfer within a hydrophobic active site, whereas in CaaD, the acidic proline is stabilized in a hydrophilic active site. The altered active site environment of CaaD thus facilitates a previously unknown reaction in the tautomerase superfamily, the hydration of the α,β -unsaturated bonds of *trans*-3-chloroacrylate and 3-bromopropiolate. The mechanism for these hydration reactions represents a novel catalytic strategy that results in carbon-halogen bond cleavage.

Dehalogenases are enzymes that cleave carbon-halogen bonds. They are found in various bacteria, allowing them to use halogenated hydrocarbons as growth substrates (1, 2). Detailed three-dimensional structural information is available for dehalogenases such as haloalkane dehalogenase, 2-haloacid deha-

logenase, and haloalcohol dehalogenase, which catalyze the cofactor-independent cleavage of the covalent bond between a halogen and an sp^3 -hybridized carbon atom by substitution mechanisms (3–5). In addition, several cofactor-dependent dehalogenases have been characterized that cleave the bond between a halogen and an sp^2 -hybridized carbon atom. Examples include heme-dependent reductive dehalogenases (6, 7) and the 4-chlorobenzoyl-CoA dehalogenases (8). In contrast, cofactor-independent dehalogenases that cleave the bond between a halogen and an sp^2 -hybridized carbon atom have only recently been discovered (9, 10).

The 3-chloroacrylic acid dehalogenases from *Pseudomonas pavonaceae* 170 represent cofactor-independent dehalogenases that catalyze the cleavage of vinylic carbon-halogen bonds, in which the halogen is bound to an sp^2 -hybridized carbon atom (9–11). They are part of a multienzyme degradation route for the *cis*- and *trans*-isomers of 1,3-dichloropropene (DCP).¹ *Cis*- and *trans*-DCP are components of the commercially produced fumigants Shell D-D and Telone II, which are used in agriculture to control plant-parasitic nematodes (10). The first step in the degradation of both DCP isomers is the hydrolytic cleavage of the bond between the chlorine and the sp^3 -hybridized carbon atom by a haloalkane dehalogenase (Fig. 1). Oxidation of the two products yields the *cis*- and *trans*-isomers of 3-chloroacrylic acid, which are subsequently dehalogenated by either the *cis*- or the *trans*-specific 3-chloroacrylic acid dehalogenase, yielding malonate semialdehyde and HCl. After enzymatic decarboxylation of malonate semialdehyde by malonate semialdehyde decarboxylase (12), the product, acetaldehyde, is likely channeled into the Krebs cycle.

The *trans*-specific 3-chloroacrylic acid dehalogenase (CaaD) from *P. pavonaceae* 170 consists of an α and a β chain having 75 and 70 residues, respectively, which both share about 25% sequence identity with the 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 (11, 13, 14). 4-OT is a trimer of homodimers of two identical 62-amino-acid chains, containing six equivalent active sites (15). Like other structurally related members of the tautomerase superfamily (16, 17), the hydrophobic active site of 4-OT harbors a catalytic N-terminal proline residue (Pro-1) with an unusually low pK_a of ~ 6.4 that functions as a proton-transferring base in a tautomerization reaction (18–23).

Biochemical characterization of CaaD uncovered a new re-

* This work was supported by The Netherlands Foundation of Chemical Research (CW) with financial aid from The Netherlands Foundation for Scientific Research (NWO) and by Grant GM-65324 from the National Institutes of Health (to C. P. W. and G. J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1S0Y) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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¹ The abbreviations used are: DCP, 1,3-dichloropropene; 4-OT, 4-oxalocrotonate tautomerase; CaaD, *trans*-3-chloroacrylic acid dehalogenase; r.m.s.d., root mean square deviation; ESI-MS, electrospray ionization mass spectrometry.

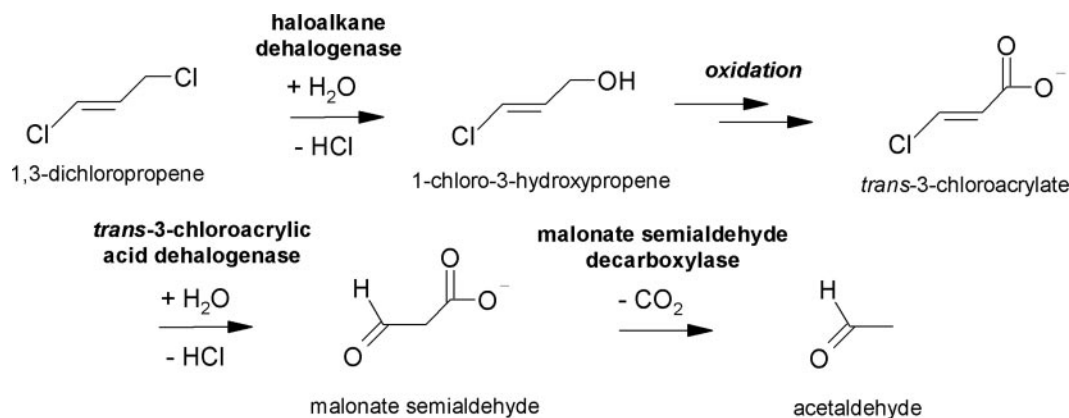


FIG. 1. The degradation of *trans*-1,3-dichloropropene in *P. pavonaceae* 170. Two dehydrogenases catalyze the oxidation of the secondary alcohol, which is formed as the result of the first dehalogenation step catalyzed by a haloalkane dehalogenase. The *trans*-3-chloroacrylic acid dehalogenase subsequently catalyzes the second dehalogenation step, after which decarboxylation yields the product acetaldehyde (10).

action specificity in the tautomerase superfamily: the dehalogenation of chloroacrylic acids (11). Although both the α and β chain of CaaD have an N-terminal proline, only β Pro-1 is essential for catalysis (11). Therefore, analogous to the oligomeric structure of 4-OT (15), CaaD was postulated to be a trimer of $\alpha\beta$ -dimers having three functional active sites, with β Pro-1 functioning as a water-activating catalytic base (11).

Here, we present the crystal structure of CaaD inactivated by 3-bromopropionate at 2.3-Å resolution. The structure suggests that a glutamate, together with the N-terminal proline, catalyzes the addition of water to the α,β -unsaturated bonds of 3-bromopropionate and *trans*-3-chloroacrylate via a Michael addition. Hydration of *trans*-3-chloroacrylate results in the formation of an unstable halohydrin intermediate, decomposition of which is accompanied by carbon-halogen bond cleavage. Dehalogenation by hydration represents a novel strategy for the cleavage of vinylic carbon-halogen bonds.

EXPERIMENTAL PROCEDURES

The CaaD mutants α R8A, α E52Q, and α E52D were constructed using the coding sequence for the dehalogenase in plasmid pET44T2 (11) as the template. The α R8A mutant was generated by PCR using the primer 5'-CACGGCATATGCGCATGATCTCTTGCACATGGCCTATGGGAGAAC-3', in which the NdeI site is in bold and the mutated codon is underlined. This primer corresponds to the 5' end of the wild-type coding sequence and was used in combination with primer R, 5'-TTGCCAAGCAGAGGGATCCCTAGCT-3', in which the BamHI site is in bold. The α E52Q and α E52D mutants were generated by overlap extension PCR (24). Primer F, 5'-CACGGCATATGCGCATGATCTCTTGCAC-3', in which the NdeI site is in bold, and primer R were used as the external primers. For the α E52Q mutant, the internal PCR primers were oligonucleotides 5'-AGCGGGATCAACTTCGTTCA-GCAGGCGAGCAT-3', in which the mutated codon is in bold, and 5'-AACGAAGTTGATCCCGCT-3' (primer A). For the α E52D mutant, the internal PCR primers were oligonucleotide 5'-AGCGGGATCAAC-TTCGTTGACCAGGCGAGCAT-3', in which the mutated codon is in bold, and primer A. The PCR reactions were carried out as described before (11), and the products were purified and cloned into plasmid pET3a (Promega Corp., Madison, WI) for overexpression of the mutant genes. The mutant genes were sequenced to verify that only the intended changes had been introduced.

Expression and purification of CaaD and its three mutants were done according to previously described procedures (11, 25). The purified mutant proteins were analyzed by electrospray ionization mass spectrometry (ESI-MS) and gel filtration chromatography as described before (25). The activity assays with the purified enzymes were performed at 22 °C by following the decrease in absorbance at 224 nm, which corresponds to the hydration of *trans*-3-chloro- ($\epsilon = 4900 \text{ M}^{-1} \text{ cm}^{-1}$) and *trans*-3-bromoacrylate ($\epsilon = 9700 \text{ M}^{-1} \text{ cm}^{-1}$) (25), or by following the release of halide using a colorimetric assay (11).

Covalently inactivated CaaD was obtained after the addition of a 25-fold excess of 3-bromopropionate to the protein solution, which was first diluted to 0.4 mg/ml to prevent overheating of the sample. The

inhibitor was synthesized by a literature procedure (26). Crystals of inactivated CaaD were obtained from 2 μ l of hanging drops consisting of equal amounts of protein solution (8 mg/ml) and well solution containing 22% (w/v) polyethylene glycol 4000 as precipitant, 100 mM sodium acetate buffer, pH 4.8, 0.15 M ammonium acetate. Plates of dimensions $1 \times 2 \times 0.2 \text{ mm}^3$ grew in a few days.

A diffraction data set to 2.3-Å resolution was collected in-house on a MacScience image plate system using Cu-K α radiation from a rotating anode generator. The data were processed using DENZO and SCALEPACK (27). The crystal diffracted to 2.3-Å resolution and had space group $P2_1$ with cell axes $a = 55.4 \text{ Å}$, $b = 100.6 \text{ Å}$, and $c = 69.9 \text{ Å}$, and $\beta = 98.9^\circ$, with two ($\alpha\beta$)₃ hexamers in the asymmetric unit.

Molecular replacement solutions were obtained with the program AMORE available in CCP4 (28, 29). A search model was constructed from the atomic coordinates of hexameric 4-OT from *P. putida* CF600 (Protein Data Bank code 1OTF (15)) by selecting for each monomer only residues 1–45, which are most similar. All residues were changed to alanines except for the proline and glycine residues. Rotation and translation functions were calculated using data between 8- and 4-Å resolution. The molecular replacement yielded the position and orientation of both hexamers in the asymmetric unit. Refinement of the solutions by AMORE gave a correlation coefficient of 0.42 and an R -factor of 49.7%. The electron density maps at this stage were not interpretable, but some density was extending from some of the alanines of the search model. A σ A-weighted map (30) calculated from the refined solutions from AMORE was used in a combined non-crystallographic symmetry averaging, density modification, and phase extension procedure using the prime-and-switch method available in the program RESOLVE (31). This improved the overall figure of merit from 0.17 after calculation of the starting map to 0.48 in the final cycle of the density modification procedure. The resulting σ A-weighted maps from RESOLVE showed improved electron density for the amino acid side chains and allowed identification of the α and β chains in the heterodimers of CaaD. Manual building of side chains and missing main chain atoms was subsequently alternated with simulated annealing and minimization runs in CNX (Accelrys Inc., San Diego, CA). RESOLVE was used to obtain the most unbiased maps for model building, until σ A-weighted $2F_o - F_c$ maps were of superior quality. The structure was built using QUANTA (Accelrys Inc.) and XtalView (32). Coordinates for the adduct were based on malonate and minimized in QUANTA (Accelrys Inc.), and its parameters were generated using the Hic-Up server (33). The quality of the final model of inactivated CaaD was analyzed with PROCHECK (34). A summary of the refinement statistics and geometric quality of the models is given in Table I. The coordinates and structure factor amplitudes of the *trans*-3-chloroacrylic acid dehalogenase CaaD have been deposited in the Protein Data Bank under accession number 1S0Y.

RESULTS AND DISCUSSION

Crystallization and Structure Elucidation—Although plate-like crystals of inactivated CaaD could easily be obtained from various acidic buffers and different polyethylene glycols as a precipitant, most of them showed smeared diffraction spots due to disorder in the plane of the crystals. Ultimately, one crystal of the inactivated enzyme was obtained that did not show these

TABLE I
Data collection and refinement statistics

	Inactivated CaaD
Data statistics	
Space group	P2 ₁
No. of hexamers/ASU (chains/ASU) ^a	2 (12)
Unit cell dimensions (Å, °)	<i>a</i> = 55.4, <i>b</i> = 100.6, <i>c</i> = 69.9 $\alpha = 90^\circ \beta = 98.9^\circ \gamma = 90^\circ$
Resolution (Å)	35–2.3
<i>R</i> _{sym} (%) overall (outer shell): 2.38–2.30 Å ^b	8.9 (27.7)
Completeness (%) overall (outer shell)	98.8 (95.2)
<i>I</i> / σ overall (outer shell)	11.0 (3.0)
Reflections total (unique)	330560 (35250)
Refinement	
Total non-hydrogen atoms/water molecules	5584/212
<i>R</i> / <i>R</i> _{free} ^c	22.1/27.4
r.m.s.d. bonds (Å)/angles (°)	0.006/1.26
r.m.s.d. B (Å ²) (main chain/side chain)	1.23/2.00
Ramachandran plot (%) (favored/allowed/ generally allowed/disallowed)	97.9/2.1/–/–

^a ASU, asymmetric unit.

^b $R_{\text{sym}} = \sum I - \langle I \rangle / \langle I \rangle$, where *I* is the observed intensity and $\langle I \rangle$ is the average intensity.

^c $R = R$ based on 95% of the data used in refinement. $R_{\text{free}} = R$ based on 5% of the data withheld for the cross-validation test.

features. After molecular replacement, the structure was built from the 2.3-Å resolution electron density maps, resulting in a well defined model of the first 60 residues of the α and β chains of the enzyme. Fifteen C-terminal residues of the α chains and 10 C-terminal residues of the β chains, accounting for 17% of the total residues, remained undefined in the electron density maps. High flexibility of these residues could provide an explanation for the disorder in the crystals.

Overall Structure of CaaD—The crystal structure of CaaD confirms that the enzyme is a trimer of $\alpha\beta$ -heterodimers. The asymmetric unit contains two such hexamers, which superimpose with r.m.s.d. values between 0.3 and 0.4 Å for 6×55 C α atoms of their core structure. The dimers are formed by the antiparallel interaction of a two-stranded parallel β -sheet of one chain with the equivalent β -sheet of the other chain, forming a four-stranded β -sheet (Fig. 2A). Two α -helices, one from each chain, lie antiparallel to each other at the concave side of the β -plane. Identical chains in the asymmetric unit superimpose with r.m.s.d. values between 0.20 and 0.35 Å for 55 C α atoms, whereas the α and β chains superimpose with r.m.s.d. values between 1.3 and 1.5 Å. Three of these $\alpha\beta$ -heterodimers, related by a non-crystallographic 3-fold rotation axis, form a barrel-like hexamer (Fig. 2B). Most dimer-dimer contacts in the hexamer are mediated by the edges of the β -sheets and are mainly of a hydrophobic nature. Each β -sheet further contributes negatively and positively charged amino acids that interact with each other in the central cavity in the hexamer (Fig. 2B). Additional dimer-dimer interactions are provided by a small β -hairpin structure at the C terminus of the α chains, extending the β -sheet of a neighboring dimer and partly covering the environment of the catalytic β Pro-1. A similar β -hairpin structure is observed in the β chains, which contributes to the environment of the catalytically inactive Pro-1 of the α chain.

The Active Site of Inactivated CaaD—CaaD contains three active sites related by the 3-fold rotation of its $(\alpha\beta)_3$ trimeric structure. They are located at the interface between two $(\alpha\beta)$ dimers on one side of the hexamer, each harboring the catalytically important N-terminal proline of the β chain (Fig. 2B). The N-terminal proline residues of the α chains are at the opposite side of the hexamer and are also related to each other by the $(\alpha\beta)_3$ 3-fold rotation. However, the environments of the

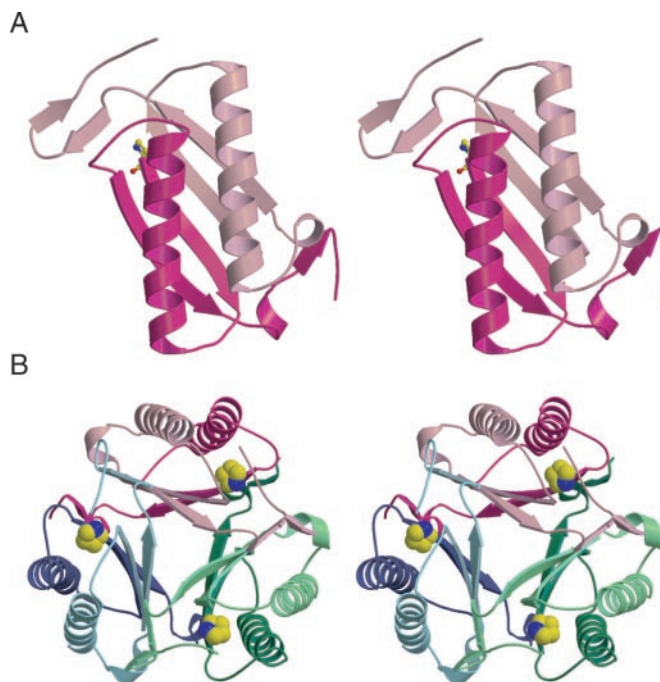


FIG. 2. Stereo views of (A) the dimeric and (B) the hexameric structure of CaaD. Dark-colored chains in each dimer represent the β chains, whereas the light-colored chains represent the α chains. The catalytically active N-terminal proline of the β chain is shown in Corey-Pauling-Koltun (CPK) representation. The figures were made using MOLSCRIPT and RASTER3D (43, 44).

α Pro-1 and β Pro-1 are different.

The catalytically active β Pro-1 is located in the interface between two $(\alpha\beta)$ dimers with several charged residues in its vicinity (Fig. 3B). Among them are the side chains of α Arg-8 and α Arg-11 from the same $\alpha\beta$ -dimer containing the catalytic β Pro-1. A third charged residue near β Pro-1 is α Glu-52 from the same α chain as α Arg-8 and α Arg-11. Its carboxylate group is fixed by the amino group of the amide side chain of β Asn-39. The residue in the α chain equivalent to the β Asn-39 (α Phe-39) also contributes to the environment of β Pro-1 by forming part of the active site wall.

Unlike β Pro-1, the catalytically inactive α Pro-1 is mostly buried from the solvent and is surrounded by mainly apolar residues. The residues near α Pro-1 that are equivalent to α Arg-8, α Arg-11, and α Glu-52 are β Ala-8, β Leu-11, and β Ile-52. These differences between the α and β chain result in a completely different environment for α Pro-1, which accounts for the observation that the N-terminal proline of the α -subunit is not able to support the dehalogenation reaction (11).

Insight into the catalyzed reaction was obtained from the analysis of the covalent adduct bound to β Pro-1 of the irreversibly inactivated form of CaaD, which resulted from the covalent modification of the enzyme by 3-bromopropiolate. The design of this compound was based on the known ability of fumarate to hydrate acetylene dicarboxylate (35). The product, hydroxyfumarate, ketonizes to afford oxaloacetate. Likewise, hydration of 3-bromopropiolate produces an unstable enol, which rearranges to an acyl bromide (25).

The electron density map of the inactivated form of CaaD demonstrates the covalent modification of only β Pro-1, and not α Pro-1. The electron density also allowed us to unambiguously establish the structure of the covalent adduct (Fig. 3B). It was first noted that none of the active sites showed electron density for a bromide ion or a bromine atom on the inhibitor. This is consistent with the previous observation of bromide release from the inhibitor upon incubation with the enzyme (25). Sec-

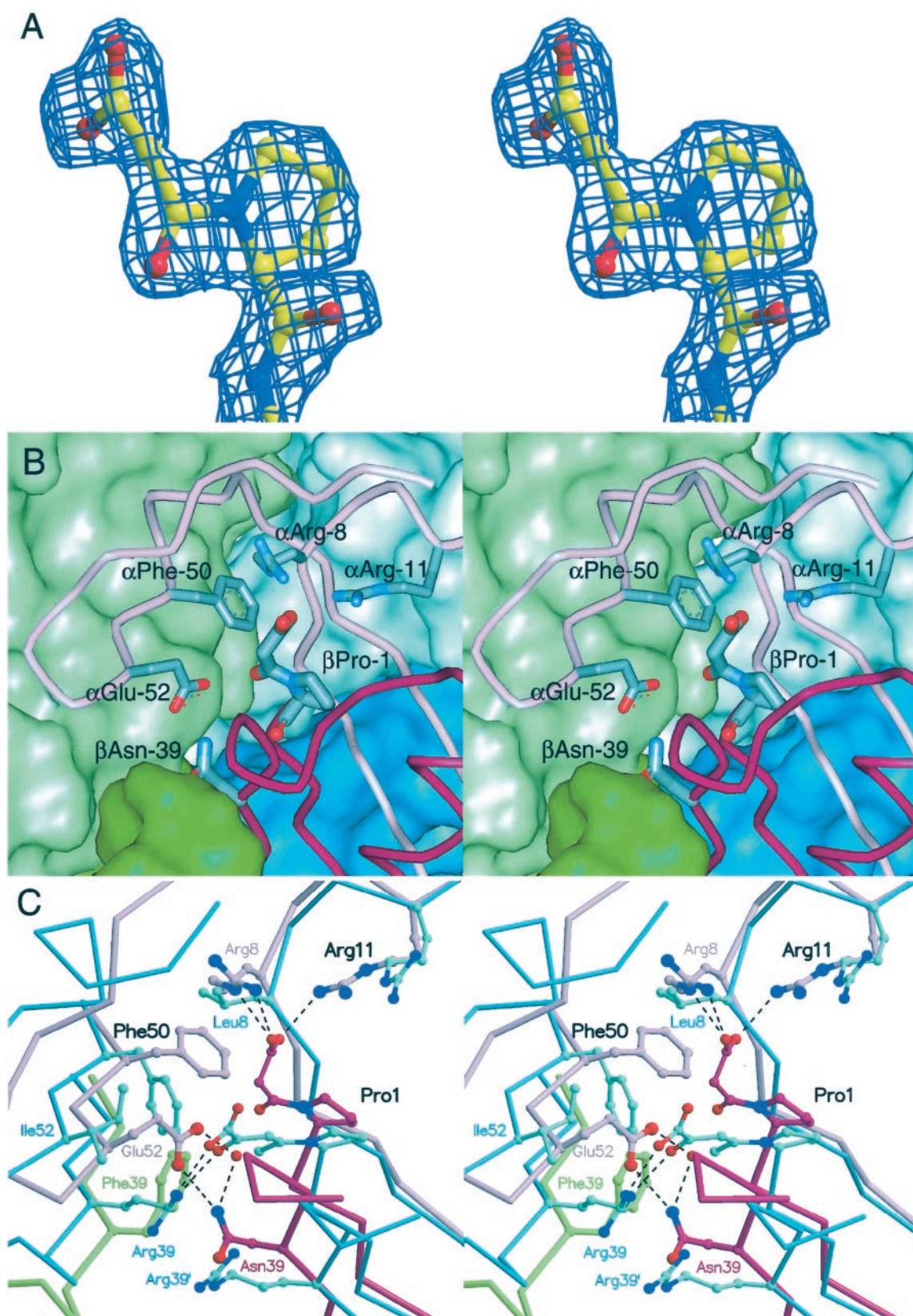


FIG. 3. **Detailed overview of the structure of inactivated CaaD.** A, stereo view of the final $2F_o - F_c$ electron density map contoured at 1.0σ from XtalView (31) covering the proline and the covalently bound adduct, clearly showing the planar peptide link to β Pro-1 and the twist of the carboxyl plane with respect to the peptide plane. B, stereo view of the active site of the inactivated enzyme showing the carboxylate group of the covalent adduct interacting with the 2 arginines. C, close-up stereo view of the superposed active site regions of inactivated CaaD and 4-OT, showing the conserved residues labeled in *black* and others labeled by their chain color. *Dark-colored chains* in each dimer represent the β chains, whereas the *light-colored chains* represent the α chains.

ond, the first two atoms attached to the prolyl nitrogen resemble a carbonyl group of a peptide bond, being in one plane with the nitrogen and carbon C α and C δ atoms of the proline ring. From this peptide, additional electron density extends toward the side chains of α Arg-8 and α Arg-11, which is compatible with a carboxylate group of the adduct. The dihedral angle between the peptide plane and the plane of the carboxylate group is 67.1°, demonstrating that no unsaturated carbon-carbon bonds are present in the adduct. On the basis of these observations, we conclude that the reaction of the enzyme with 3-bromopropionate has resulted in the attachment of a malonyl group to the nitrogen atom of the N-terminal proline (Fig. 3, *a* and *b*). This result is in agreement with the previously reported mass increase of the β chain upon covalent modification (25). The modification of the β Pro-1 by a malonyl group may at first seem surprising but is readily explained by the addition of a water molecule to the carbon-carbon triple bond as a first step in the reaction of 3-bromopropionate with the enzyme (see below).

Apart from the interaction of its carboxylate group with α Arg-8 and α Arg-11, the adduct further has hydrophobic contacts with α Phe-50, α Leu-57, and β Ile-37. Interestingly, one active site in each of the two different ($\alpha\beta$)₃ hexamers in the asymmetric unit shows electron density for a water molecule between the α Glu-52 O ϵ_2 atom and the nitrogen atom of the modified proline. Strikingly, the covalent adduct in this active site shows negative electron density at the position of its carboxylate group. Because β -ketoacids readily undergo decarboxylation (36), the binding of the water molecule likely correlates with the presence of an acetylated proline resulting from decarboxylation of the malonyl group. This observation is also consistent with previous mass spectral analysis showing decarboxylation of the malonyl adduct (25). In the active sites that lack a bound water molecule, the density for the carboxylate group is fully present, indicating that decarboxylation had not occurred. In these active sites, the α Glu-52 O ϵ_2 atoms make a hydrogen bond of \sim 2.9 Å with the backbone carbonyl group of β Ile-37, suggesting that the glutamate side chain is protonated.

Mutagenesis of α Arg-8 and α Glu-52—On the basis of sequence analysis and site-directed mutagenesis studies, β Pro-1 and α Arg-11 were identified previously as critical residues in CaaD (11, 25). The crystallographic results suggest that α Arg-8 and α Glu-52 may also play important roles in the mechanism. Therefore, three single site-directed mutants were constructed in which α Glu-52 was replaced with a glutamine (α E52Q) or aspartate (α E52D) and α Arg-8 was replaced with an alanine (α R8A). The three mutants were expressed in *Escherichia coli* BL21(DE3) and purified to \sim 95% homogeneity (as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using the protocol described for wild-type CaaD (25). Analysis of the mutant proteins by ESI-MS and gel filtration chromatography indicated that the α - and β -subunits had the expected masses (and were not blocked by the initiating methionine) and that the mutants were heterohexamers in solution.

In a spectrophotometric assay, which measures the depletion of substrate at 224 nm, activity was not detected for any of the mutants using *trans*-3-chloro- and *trans*-3-bromoacrylate (after 30 min). However, a 24-h incubation period of the mutant proteins (in separate reactions) with *trans*-3-chloroacrylate showed that the α E52D and α R8A mutants retained a small amount of activity (as determined by a colorimetric assay that monitored the release of the halide), whereas the α E52Q mutant had no detectable activity. Based on these results, we conclude that α Arg-8 and α Glu-52 are indeed important for the CaaD-catalyzed hydration reactions.

Similarity to Other Members of the 4-OT Family—The crys-

tal structures of the irreversibly inactivated CaaD and 4-OT (37) superimpose with an r.m.s.d. of 2.1 Å for 6 \times 50 C α atoms/hexamer. The α and β chains of CaaD superimpose on the single chain that builds the 4-OT homohexamer with r.m.s.d. values of \sim 1.3 and 1.6 Å, respectively, for 55 C α atoms. The high r.m.s.d. values are mainly due to deviations in the regions that contribute to the active site environment of β Pro-1, whereas most of the β - α structural core of the $\alpha\beta$ -dimers superimposes nearly perfectly. In particular, the C-terminal β -hairpin structures and the loop regions between residues 35 and 41 in both chains are shifted in the direction of β Pro-1 with respect to their equivalents in 4-OT, resulting in a more narrow active site cavity in CaaD. This is not surprising since *trans*-3-chloroacrylate is considerably smaller than the elongated 2-oxo-4-*trans*-hexenedioate substrate of 4-OT.

Comparison of the inhibited CaaD and 4-OT structures (Fig. 3C) shows that the inhibitors interact differently with the enzymes. In 4-OT, Arg-39 binds the keto and carboxylate groups of the inhibitor, whereas in CaaD, Arg-39 is replaced by α Phe-39 and β Asn-39. Instead, α Arg-8 and α Arg-11 interact with the carboxylate group of the inhibitor. Arg-11 is also present in 4-OT, where it likely interacts with the C-6 carboxylate group of 2-oxo-4-*trans*-hexenedioate (21). Thus, of the 2 arginines that bind the substrate in 4-OT, only α Arg-11 is conserved in CaaD.

The most striking difference between CaaD and 4-OT is the presence of α Glu-52 at 4.5–6.0 Å from β Pro-1. In 4-OT, an isoleucine is present at the equivalent position, 8–9 Å away from the N-terminal proline. In addition, Phe-50 contributes to the hydrophobic active site environment of Pro-1 in 4-OT; α Phe-50 is also present in CaaD. In both enzymes, these residues are part of the C-terminal β -hairpin structure that covers the active site pocket. Although the hydrophobic residues of the β -hairpin are important for the low pK_a of the Pro-1 of 4-OT by preventing water from entering the active site (23), the corresponding β -hairpin glutamate in CaaD creates a more hydrophilic active site environment. A negatively charged residue near the N-terminal proline is unprecedented in the tautomerase superfamily (14, 15, 38) and has important consequences for the role of β Pro-1 in the catalytic mechanism of CaaD as well as for the mechanism of inactivation of CaaD by 3-bromopropionate.

Mechanism of Inactivation of CaaD by 3-Bromopropionate—CaaD is well equipped to catalyze the addition of water to the triple bond of 3-halopropionates, which are analogues of the substrates for CaaD. The specific interaction of the covalent malonyl adduct with the 2 arginines suggests the favored orientation of 3-bromopropionate in the active site. The bromine atom may bind deeply in the active site pocket, possibly interacting with the 2 phenylalanines that constitute the active site wall. In this binding mode, the C-2 carbon atom would be close to the prolyl nitrogen of β Pro-1, whereas the C-3 carbon atom of 3-bromopropionate would be positioned close to the water molecule hydrogen-bonded to α Glu-52 and β Pro-1 as seen in the active site with the (partly) acetylated proline. The glutamate side chain interacts with the amino group of the side chain of β Asn-39, the bridging water molecule, and a second surface-bound water molecule, suggesting that its side chain carboxylate group is likely stabilized in a charged anionic form. The water-mediated interaction of the charged glutamate with the prolyl nitrogen of β Pro-1 suggests that the proline is protonated at physiological pH, which is consistent with its position in a hydrophilic environment. In contrast to the Pro-1 catalytic base in 4-OT (23), β Pro-1 may thus function as a general acid that protonates the C2 carbon atom of the inhibitor (Fig. 4A). The anionic α Glu-52 could act as the general base that acti-

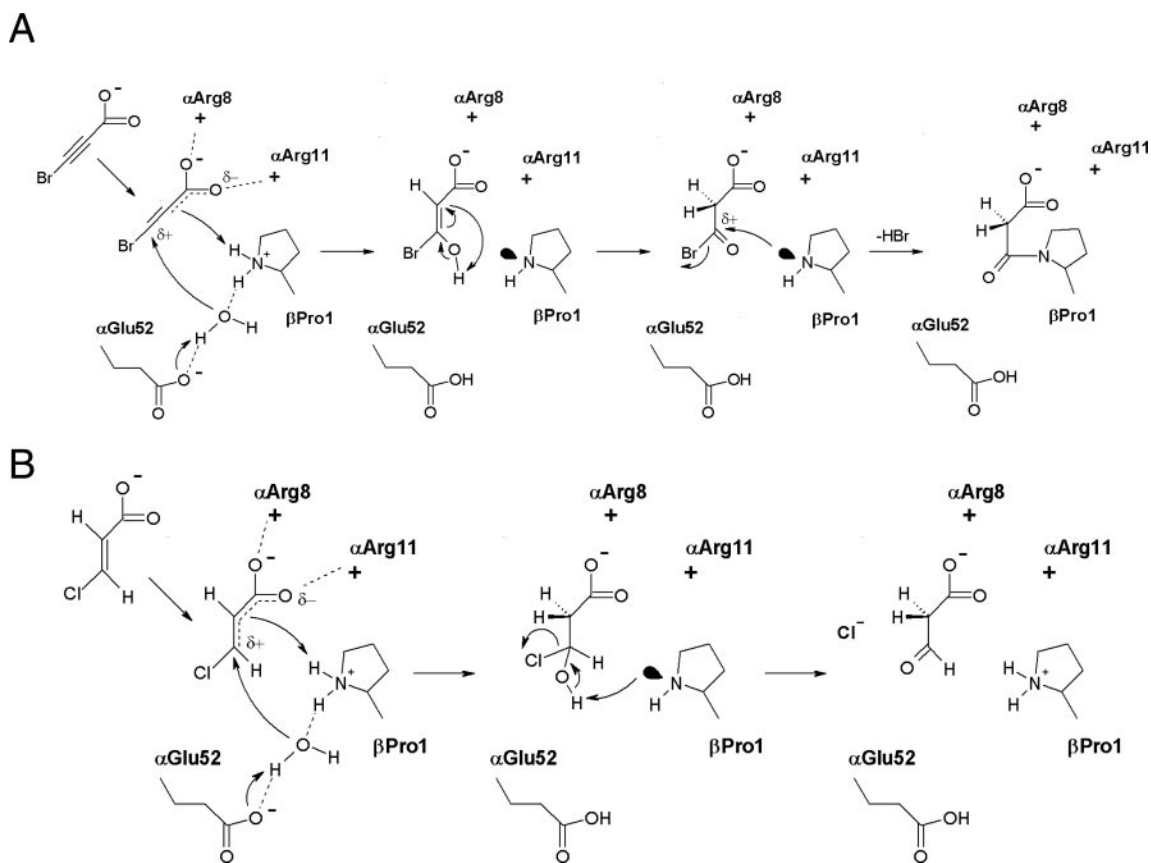


FIG. 4. **The catalytic mechanism of CaaD.** A, schematic representation of the proposed mechanism of inactivation of CaaD by 3-bromopropiolate, which results from the formation of a reactive acyl bromide intermediate that is produced by hydration of the substrate analogue. B, schematic representation of the proposed dehalogenation mechanism of *trans*-3-chloroacrylate by CaaD, in which hydration of the substrate yields an unstable halohydrin that subsequently collapses to yield malonate semialdehyde and HCl. The exact distribution of the charges over the atoms of 3-bromopropiolate and *trans*-3-chloroacrylate is not known.

vates a water molecule for a nucleophilic attack at the C-3 position. The arginine residues, αArg-8 and αArg-11, favor the formation of a partial positive charge at C-3 by polarizing the carboxylate group of the inhibitor, thereby increasing the susceptibility of the C-3 position for nucleophilic attack. Strikingly, the glutamate is hydrogen-bonded to the backbone carbonyl function of βIle-37 in the majority of the active sites of the inactivated enzyme. This suggests that the side chain carboxylate group has been protonated during the inactivation reaction, further supporting its role as the water-activating base.

Hydration of the triple bond in 3-bromopropiolate would thus yield 3-bromo-3-hydroxypropenoate, which rapidly rearranges to yield malonyl bromide (Fig. 4A). The acyl bromide function is very reactive toward nucleophiles (39). Because the prolyl nitrogen of βPro-1 has lost its proton in the hydration step, it may now function as a nucleophile and react with the acyl bromide, resulting in the formation of the covalent malonyl adduct and the departure of a bromide ion (25). It was proposed previously that inactivation of CaaD by 3-bromopropiolate could occur by this mechanism-based route to produce an acyl bromide (or a ketene). Alternatively, a Michael addition of βPro-1 to C-3 of 3-bromopropiolate was proposed (25). The crystallographic results strongly support the mechanism-based route because the active site is clearly set up to carry out a hydration reaction and the protonated βPro-1 is not sufficiently nucleophilic to attack 3-bromopropiolate in a Michael fashion. Thus, 3-bromopropiolate is a mechanism-based inhibitor of CaaD.

Conversion of *trans*-3-Chloroacrylate by CaaD—The geometry of the inactivated complex, the mechanism proposed for inactivation, and the results of the site-directed mutagenesis experiments suggest a mechanism for the dehalogenation of

trans-3-chloroacrylate by CaaD. In the catalytic mechanism, αGlu-52 and βPro-1 can catalyze the Michael addition of water to the double bond of *trans*-3-chloroacrylate (Fig. 4B). The 2 arginines likely polarize the carboxylate group, thereby stabilizing a partial positive charge on the C-3 carbon atom. This facilitates nucleophilic attack by the water molecule and the subsequent transfer of a proton from βPro-1 to the C-2 carbon atom. The addition of water results in the unstable 3-chloro-3-hydroxypropanoate intermediate, which subsequently decomposes to yield malonate semialdehyde and HCl. The cleavage of the carbon-chlorine bond in the chlorohydrin intermediate is now a chemically favorable reaction that may not need to be further catalyzed by specific halide-binding residues (35). There is, however, a possibility that the N-terminal proline residue plays an active role in the collapse of 3-chloro-3-hydroxypropanoate by deprotonating its hydroxyl group. In this manner, the proline would also regenerate its initially charged form, thereby returning the system to its native state to enable another turnover. Thus, dehalogenation of *trans*-3-chloroacrylate by CaaD is mechanistically a hydration reaction, which represents a novel strategy for the cleavage of a bond between a halogen and an *sp*²-hybridized carbon atom.

An alternate mechanism involves the formation of a carbocation intermediate. In this scenario, βPro-1 initiates the reaction by providing a proton to C-2, which generates a positive charge at C-3. The nearby water molecule, held in position by αGlu-52, adds to the carbocation to afford the halohydrin intermediate. In this mechanistic scenario, the 2 arginines are more likely to play a role in substrate alignment. Although carbocation intermediates are rare in enzyme-catalyzed reactions, such a possibility cannot be ruled out.

The Evolutionary Origin of Chloroacrylic Acid Dehalogenating Activity—Although other structurally characterized members of the tautomerase superfamily contain homodimers or fused heterodimers in different stoichiometries (14–17), CaaD is built up of a trimer of $\alpha\beta$ -dimers of two different tightly packed peptide chains. The α and β chains form three identical active sites that carry out the dehalogenation of *trans*-3-chloroacrylate by hydration. Although most superfamily members known so far use the conserved N-terminal proline as a proton-transferring catalytic base in tautomerization reactions, the β Pro-1 of CaaD functions as a general acid in a hydration reaction.

Surprisingly, 4-OT and YwhB, a 4-OT homologue in *Bacillus subtilis*, have a rudimentary *trans*-3-chloroacrylic acid dehalogenase activity, in addition to their major tautomerase activities (45). Such catalytic promiscuity has also been observed in other enzyme superfamilies, suggesting that these secondary activities could play a role in the evolution of new enzymatic activities (40). The low level dehalogenase activity of 4-OT could result from the interaction of Arg-11 with the carboxylate group to create a partial positive charge at C-3, thereby facilitating a Michael addition of water to *trans*-3-chloroacrylate. The catalytic proline could assist in catalysis by activating the water molecule. The efficiency of the dehalogenation reaction might have been limited by the inability of the enzyme to carry out a hydration reaction in a hydrophobic pocket. Moreover, the presence of water in the active site could raise the pK_a of the proline, thereby decreasing its effectiveness as a base. Evolutionary selection may ultimately have led to the altered active site environment that enabled a more efficient hydration of the substrate.

A BLAST search (41) against the protein sequence databases yields various (hypothetical) 4-OT sequences. Only one of them shares the characteristic glutamate and 2 arginines that are present in CaaD, suggesting that this variation in the tautomerase superfamily is rare. The gene (*cg10062*), encoding a 149-amino-acid protein corresponding to a fused α and β chain of CaaD, occurs in the genome of *Corynebacterium glutamicum* ATCC 13032 (42), a bacterium that is widely used in the amino acid fermentation industry. Interestingly, this gene product exhibits a low level dehalogenase activity toward *cis*-3-chloroacrylate.² This may be another example of a hydratase or tautomerase with promiscuous dehalogenase activity.

Acknowledgments—We thank Dr. William H. Johnson, Jr. for synthesizing 3-bromopropionic acid. We also thank Hector Serrano for assistance in purifying the CaaD mutants.

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² G. J. Poelarends, H. Serrano, and C. P. Whitman, unpublished data.